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Subgingival Lipid A Profile and Endotoxin Activity in Periodontal Health and Disease

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Key words: Lipopolysaccharide, lipid A, subgingival microbiota, biomarker, periodontal diseases.

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ABSTRACT

Objectives: Regulation of lipopolysaccharide (LPS) chemical composition, particularly its lipid A domain, is an important, naturally occurring mechanism that drives bacteria-host immune system interactions into either a symbiotic or pathogenic relationship. Members of the subgingival oral microbiota can critically modulate host immuno-inflammatory responses by synthesizing different LPS isoforms. The objectives of this study were to analyse subgingival lipid A profiles and endotoxin activities in periodontal health and disease and to evaluate the use of the recombinant factor C assay as a new, lipid A-based biosensor for personalized, point-of-care periodontal therapy.

Materials and Methods: Subgingival plaque samples were collected from healthy individuals and chronic periodontitis patients before and after periodontal therapy. Chemical composition of subgingival lipid A moieties was determined by ESI-Mass Spectrometry. Endotoxin activity of subgingival LPS extracts was assessed using the recombinant factor C assay, and their inflammatory potential was examined in THP-1-derived macrophages by measuring TNF-α and IL-8 production.

Results: Characteristic lipid A molecular signatures, corresponding to over-acylated, bi-phosphorylated lipid A isoforms, were observed in diseased samples. Healthy and post-treatment samples were characterized by lower *m/z* peaks, related to under-acylated, hypo-phosphorylated lipid A structures. Endotoxin activity levels and inflammatory potentials of subgingival LPS extracts from periodontitis patients were significantly higher compared to healthy and post-treatment samples.

Conclusions: This is the first study to consider structure-function-clinical implications of different lipid A isoforms present in the subgingival niche and sheds new light on molecular pathogenic mechanisms of subgingival biofilm communities.

Clinical Relevance: Subgingival endotoxin activity (determined by lipid A chemical composition) could be a reliable, bacterially-derived biomarker and a risk assessment tool for personalised periodontal care.

INTRODUCTION

Personalised healthcare is beginning to achieve its goal of delivering the right therapy to the right patient at the right time. The wave of predictive, preventive and personalised medicine is rapidly influencing decisions made with regard to prevention, early diagnosis and treatment of oral diseases [1]. Chronic periodontitis is an irreversible, multifactorial, inflammatory disease of the tissues supporting the teeth and is caused by a disruption to normal homeostatic mechanisms by numerous bacterial species found in subgingival dental plaque [2].

The causative basis for chronic periodontitis relates to an excessive host innate immune response to a unique bacterial consortium and the virulence factors produced by certain pathogenic members of the subgingival biofilm community [3]. Lipopolysaccharide (LPS) is a key microbial stimulus that triggers the host response in periodontal tissues [4]. LPS is the major constituent of the outer membrane of Gram-negative bacteria where it plays an important structural role and mediates interaction between bacteria and the environment [5]. LPS is composed of hydrophilic O-antigen polysaccharides and an amphipathic lipid A anchor which is incorporated into the outer bacterial cell membrane. Lipid A, the bioactive centre of LPS, is considered to be an archetypal microbe-associated molecular pattern (MAMP) molecule [6]. Multicellular organisms, from horseshoe crabs and fruit flies to humans, have evolved proteins specialized for the recognition of LPS. In horseshoe crabs, Factor C is an effective biosensor, which alerts the horseshoe crab to the presence of a Gram-negative invader, while in humans, phagocytic cells recognize non-self motifs through an extensive repertoire of evolutionary conserved Toll-like Receptors (TLRs) [7]. Activation of TLRs by lipid A in macrophages triggers intracellular signalling cascades that lead to secretion of chemokines and pro-inflammatory cytokines responsible for the development of clinical signs of inflammation and, in the case of periodontitis, alveolar bone loss [8].

TLRs and Factor C can be activated by structurally diverse lipid A molecules. Minor changes in the chemical composition of lipid A can affect LPS's inflammatory potential. The potency of TLR activation decreases in line with a decrease in the number of fatty acids and phosphate groups attached to the lipid A diglucosamine backbone [9]. The subgingival biofilm community has the potential to modulate TLR activity and subsequently subvert intricate homeostatic mechanisms involved in the maintenance of periodontal health, by the expression of different LPS isoforms [10]. Tetra- and penta-acylated lipid A structures of *Porphyromonas gingivalis* LPS have been shown to differentially activate TLR4-mediated NF-κB signaling pathway in human gingival fibroblasts and critically modulate the expression of pro-inflammatory cytokines [11].

The elucidation of this pathogenic mechanism in relation to lipid A structure-function-clinical implications is central to the understanding of host-microbial interaction at periodontally healthy and diseased sites. Therefore, in this study we examined structural diversity of subgingival lipid A isoforms in healthy and diseased patients (before and after periodontal therapy) and evaluated the Factor C based assay and its interaction with subgingival LPS extracts as a bacterially derived biomarker for prevention, early intervention and patient-centred management of chronic periodontitis.

MATERIALS AND METHODS

Study population

Approval of the study protocol was obtained from the Health Research Authority, UK (NRES Committee South West - Cornwall and Plymouth 14/SW/0020) and the study was conducted in accordance with the Helsinki Declaration of 1975, as revised in 2013. All participants provided written consent. Thirty-two individuals (11 female, 21 male, mean age 46) with chronic periodontitis (CP) and 33 systemically and periodontally healthy (H) persons (18

female, 15 male, mean age 31) were recruited from patients presenting to the Peninsula Dental School, University of Plymouth, UK.

CP patients were diagnosed in accordance to the accepted clinical criteria [12]: a minimum of 4 teeth in each jaw with a probing depth (PD) of ≥ 5 mm; bleeding on probing and clinical attachment level (CAL) of ≥ 4 mm; with ≥ 50% alveolar bone loss in at least two quadrants (assessed radiographically). Periodontal health was defined as ≥ 90% of the measured sites with PD < 3 mm and no bleeding on probing (BOP). Clinical parameters (six-point pocket chart, plaque index (PI) and bleeding index (BI)) and demographic characteristics of the study population were recorded as well as their smoking status (3 smokers in the healthy group and 14 in CP) (Table 1.). Patients with medical disorders unrelated to periodontal disease, but likely to influence study outcomes, or with antibiotic or periodontal treatment in the previous 6 months were excluded from the study.

Insert Table 1.

Subgingival biofilm collection

Subgingival biofilm samples were collected by inserting sterile paper points for 30 seconds in three deepest bleeding pockets in CP patients and in healthy patients from three non-bleeding sites (mid-labial of UR1, mesio-buccal of UR6 and mesio-buccal of LL6) and were pooled for each individual patient. Diseased patients underwent conventional, non-surgical periodontal therapy and subgingival biofilm samples were collected from the same sites, three months after the completion of the therapy.

LPS extraction and lipid A isolation

LPS from subgingival samples was extracted using the LPS extraction kit (iNtRON Biotechnology, S.Korea) following the manufacturer's instructions**.** Extracted LPS was re-

suspended in 500µl of LPS-free water and stored at 4ºC. For lipid A isolation, all healthy, diseased and post-treatment samples were pooled and lipid A was isolated by mild hydrolysis as described by Coats et al. [13].

Mass-spectrometry

Lipid A was desalted with 0.1M ammonium citrate and dissolved in methanol/dichloromethane (3:1, v/v). Mass spectrometric analysis of lipid A was performed on a 6530 Accurate Mass Quadrupole Time-Of-Flight (Q-TOF) MS system (Agilent Technologies, Singapore). Positive and negative ion mass spectra were recorded over the range of 1000–2100 *m/z*. The electrospray ion source (ESI) was operated using the following conditions: pressure of nebulizing gas (N2) was 30 psi; temperature and flow rate of drying gas (N2) were 300°C and 7 L/min, respectively; temperature and flow rate of sheath gas were 300°C and 11 L/min, respectively. The capillary voltage was set to 3.5 kV, the nozzle voltage to 2 kV, the fragmentor potential to 100 V and the skimmer potential to 65 V.

Endotoxin activity (Factor C assay)

The *Limulus amebocyte* lysate (LAL) alternative, recombinant Factor C assay (rFC), is a representative of modern recombinant methods, moving away from the animal source, which for decades has been the horseshoe crab. The endotoxin-specificity of the Factor C assay eliminates all false-positive and elevated results seen by ß-glucan reactions in the LAL test. Subgingival LPS extracts were diluted 1:10 in endotoxin free water and endotoxin activity was measured according to manufacturer's instructions (EndoZyme, Hyglos, Germany).

Inflammatory potential of subgingival LPS isolates

For THP-1 differentiation into macrophages, the cells were plated at a density of 1×10^6 cell/ml in complete RPMI medium (Lonza, UK) and differentiated with PMA (25 ng/ml) for 3 days followed by 24h resting in fresh medium [14].

Cells were stimulated with 50 µl of subgingival LPS extracts per ml of cells for 18 hours. Cell free supernatants were collected and concentrations of IL-8 and TNF-α were measured by ELISA (BD Bioscience, UK).

Statistical Analyses

All samples, participants and clinical data were anonymised and locked before the codes were revealed. In order to analyse differences between examined groups of patients, one-way analysis of variance with Tukey post-hoc test was performed, using GraphPad Software, San Diego, CA. Correlations between clinical parameters and LPS endotoxin activity and inflammatory potential were analysed using the Pearson correlation coefficient. A p value below 0.05 was considered significant (*<0.05; **<0.01; ***<0.001). All experiments were performed at least three times in duplicates.

RESULTS

Structural characterization of subgingival lipid A isoforms

Characteristic lipid A molecular signatures were observed in the spectra of LPS extracts from the three groups of patients. Mass-spectrometry analyses of lipid A isolated from pooled healthy (H) and chronic periodontitis (CP) subgingival plaque samples revealed the presence of prominent, high *m/z* peaks (*m/z* between 1600-1900) in diseased samples, which were not present in the healthy group (Fig. 1(b) and 1(c)). These high *m/z* values are consistent with more phosphorylated and over-acylated lipid A isoforms as it is the case with reported *P.gingivalis* LPS (Fig. 1(a)). Interestingly, in subgingival samples taken from the same sites, three months after the completion of periodontal therapy, these ions were not detectable (Fig. 1(d)) and the lipid A mass spectrum of the pooled post-treatment samples resembled the

spectrum of healthy individuals (especially in the positive-ion mode), consistent with less-acylated, hypo-phosphorylated lipid A isoforms .

Endotoxin activity of subgingival biofilm samples

Endotoxin activity of subgingival LPS extracts was measured for each individual patient using the recombinant factor C assay. LPS extracts from chronic periodontitis patients showed significantly higher levels of endotoxin activity compared to healthy individuals (Fig. 2). Most healthy patients' endotoxin activity was below the level of 10 EU while in a significant number of diseased patients (19) this value exceeded 20 EU. Smoking status did not appear to significantly influence the level of subgingival endotoxin activity, but there was a trend of lower values in diseased smokers (data not shown). Endotoxin activity of samples taken after periodontal treatment was significantly lower compared to the corresponding CP samples, with values at a similar range as for healthy individuals. Interestingly, in all three groups of samples there were two distinct sub-groups; one in the lower range of EU where most of the healthy and post-treatment samples were aggregated and one within a higher range of endotoxin activity where most of the CP samples congregated. These two sub-groups may represent differences in disease activity and progression, two of the disease signatures currently unmeasurable at point-of-care.

The diagnostic performance of subgingival endotoxin activity as a biomarker for disease status was evaluated using the receiver operating curve (ROC) which demonstrated a good predictable value of endotoxin activity levels (area under the curve (AUC) of 0.94) and the optimal cut-off point of 5.2 EU (sensitivity 90.63% and specificity 87.50%) (Fig. 3).

Inflammatory potential of subgingival LPS extracts

Inflammatory potential of subgingival LPS extracts from each patient was assessed in macrophages derived from THP-1 cells, by measuring the production of TNF-α and IL-8. The cytokine values were also correlated with corresponding endotoxin activity levels. TNF-α production by macrophages challenged with LPS extracts from most of the healthy individuals was undetectable while LPS extracts from diseased patients triggered significantly higher production of TNF-α ($p<0.001$) (Fig. 4(a)). There was a trend of decreased TNF-α production by macrophages challenged with post-treatment LPS extracts. Similarly, IL-8 production was significantly greater in macrophages treated with diseased LPS extracts compared to both healthy and post-treatment samples (Fig. 4(b)). In addition, diseased samples triggered a wider range of TNF-α and IL-8 levels compared to healthy and post-treatment samples. Moreover, significantly higher levels of IL-8 than TNF-α were observed in the healthy group, depicting the importance of leukocytes transmigration for periodontal homeostasis.

There was a strong positive correlation between endotoxin activity levels of subgingival LPS extracts and their inflammatory potential measured by the production of TNF-α and IL-8 ($p<0.001$) (Fig. 4(c) and 4(d)).

DISCUSSION

The distinction between periodontopathogens and oral commensals is becoming increasingly blurred. Periodontal diseases are caused by the action of bacteria that are also present in health and that involves complex host–microbe interactions [15]. An omics approach to periodontal diseases' aetiopathogenesis can potentially be used to detect microbial factors responsible for subclinical alterations in tissue metabolism, inflammatory cell recruitment and bone remodelling before extensive clinical damage has occurred [16].

Lipopolysaccharide is an essential microbial macromolecule found on the outer surface of Gram-negative bacteria, which plays a critical role in maintaining bacterial structural integrity and establishing selective permeability of the bacterial cell wall [17]. LPS from periodontopathogenic bacteria, and particularly its lipid A domain, is recognized as a foreign molecule by the human host and is crucial for triggering an inflammatory response in periodontal tissues [18]. The ability of *P.gingivalis* to modify the structure of its lipid A moieties is critical for establishing dysbiotic subgingival biofilm communities associated with periodontal diseases in an animal model [19]. The LPS structure and immunogenicity of other periodontal pathogens, *T. forsythia*, *A. actinomycetemcomitans* and *F.nucleatum* have been published but the correlations between lipid A chemical composition and the disease status and progression remains elusive [20] [21] [22] .

This is the first study to examine lipid A compositions of LPS isolated directly from subgingival biofilm samples and not from cultured bacteria. We show here that there are significant structural differences between lipid A isoforms isolated from subgingival plaque samples from healthy individuals and patients with chronic periodontitis. MS analysis revealed the presence of peaks at a higher *m/z* ratio in CP patients that correspond to more phosphorylated and over-acylated lipid A isoforms. These peaks were not observed in healthy patients or in CP patients after periodontal treatment. There is strong evidence that the expression of highly phosphorylated and acylated lipid A isoforms is an important virulence factor of many prominent human pathogens. John *et al*. have shown that lipid As of all *N. meningitis* invasive isolates are hexa-acylated whereas lipid As of significant number of carrier strains are penta-acylated [23]. *P.aeruginosa* from cystic fibrosis patients is able to synthesise a unique hepta-acylated lipid A moiety, not normally present in environmental isolates, that significantly contributes to the overall inflammatory burden of the airways of these patients [24]. In contrast, *H.pylori* modifies its lipid A by removal of phosphate groups from the 1- and 4′-positions of the lipid A backbone producing under-acylated and under-phosphorylated lipid

A isoforms which are characterised by strikingly low endotoxicity. These modifications decrease the recognition of this bacterium by TLR4 and are key to its ability to colonize a mammalian host [25].

Previous studies have shown that periodontal therapy leads to a rapid reduction in periodontal pathogens and a slower reduction in other species that can be sustained for at least 2 years [26]. Our results complement these findings with the fact that not only is the composition of subgingival microflora different between healthy and diseased patients but also that there are significant differences in microbial structural components at a subspecies level. Further studies are needed to determine the dynamics and stability of these changes in longer terms.

One of the limitations of our study is that the patient in the healthy group were younger than the periodontitis patients. The influence of ageing on the lipid A structure is not known but the long-term stability of the oral microbiome and the similarity of subgingival microbiota composition between older adults and youngsters with periodontal health and periodontitis have been documented [27].

The recombinant factor C assay (rFC) is widely used for the screening of lipopolysaccharide in pharmaceutical products and can detect a wide range of divergent LPS structural species [28]. The minimal lipid A structural prerequisites for the activation of factor C is the presence of the 4'- phosphate-diglucosamine backbone. However, full expression of factor C activity is also dependent on the presence of fatty acids attached to this backbone, and interestingly does not depend on the type of LPS aggregate structure [29]. The ratio of high endotoxic and low endotoxic LPS is crucial for the regulation of the intestinal immune balance, where high endotoxic LPS isoforms support intestinal inflammation while low endotoxic LPS promotes intestinal homeostasis [30]. Our results show significantly increased endotoxin activity of subgingival plaque samples from CP patients compared to healthy individuals and treated CP patients. The differences in endotoxin activity are in agreement with different lipid A isoforms

observed in these three groups of samples. Subgingival endotoxin activity levels exhibited a high level of sensitivity, specificity and accuracy in confirming the clinical classification of chronic periodontitis status.

The destruction of alveolar bone and periodontal ligament is caused by the ability of subgingival microbial community to induce a dysregulated inflammatory response in periodontal tissues [31]. Subgingival plaque samples from diseased sites have been shown to strongly activate TLR4, whereas matched samples obtained from healthy sites have been more variable with some of them displaying strong TLR4 antagonism [12]. In our study, inflammatory potential of subgingival LPS extracts, measured by the production of TNF-α and IL-8 in THP-1 differentiated macrophages, was significantly higher in diseased patients compared to healthy individuals for TNF-α, and between diseased and both healthy and post-treatment samples for IL-8. In addition, a strong, positive correlation between subgingival endotoxin activity levels and inflammatory potentials of subgingival LPS extracts supports a potential use of subgingival endotoxin activity measurements as a biomarker for the development of inflammatory periodontal conditions.

This is the first study to consider structure-function-clinical implications of different lipid A isoforms present in the subgingival niche and sheds new light on molecular pathogenic mechanisms of subgingival biofilm communities. The results of this study may influence the development of a novel, lipid A based, chair-side test for personalised, point-of-care decision making and new approaches to the treatment of periodontal diseases by manipulating bacterial ability to modify their LPS. Further studies are needed to confirm the presence of lipid A species within periodontal tissues and include gingivitis patients.

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Compliance with Ethical Standards

Conflict of Interest: SZ has a patent on periodontitis biomarkers, publication number GB2549712. Author AS declares that he has no conflict of interest. Author ZH declares that she has no conflict of interest. Author CM declares that she has no conflict of interest. Author MJ declares that he has no conflict of interest. Author LB declares that she has no conflict of interest. Author AK declares that she has no conflict of interest. Author AF declares that he has no conflict of interest. Author SJ declares that he has no conflict of interest.

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Ethical approval: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee

and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent: Informed consent was obtained from all individual participants included in the study.

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TABLES:

Table 1: Summary of clinical characteristics of the study population (means ± SD).

	Healthy n=33	Periodontitis n=32	
		Baseline	Post-treatment
Age (years)	31 ± 9	46 ± 9	46 ± 9
O'Leary Plaque Index (%)	14.68 ± 5.02	56.65 ± 21.53	33.92 ± 14.20
Bleeding Index (%)	2.22 ± 2.76	41.47 ± 18.49	23.06 ± 13.31
Probing Depth (mm)	1.45 ± 0.45	5.78 ± 0.61	4.47 ± 0.58

FIGURES:

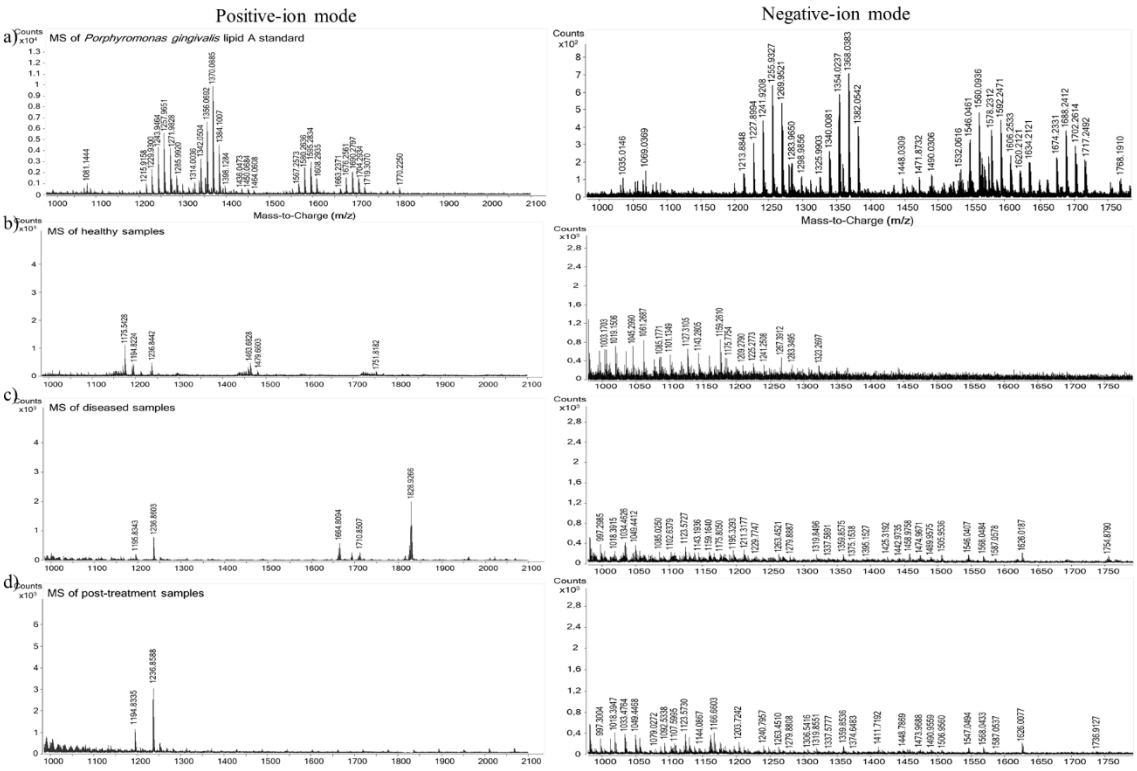

Fig. 1 Illustrative electrospray ionisation mass spectra of lipid A isolated from a) *P.gingivalis* LPS (InvivoGen); and pooled subgingival plaque samples obtained from b) healthy individuals; c) chronic periodontitis patients; and d) chronic periodontitis patients three months after periodontal therapy.

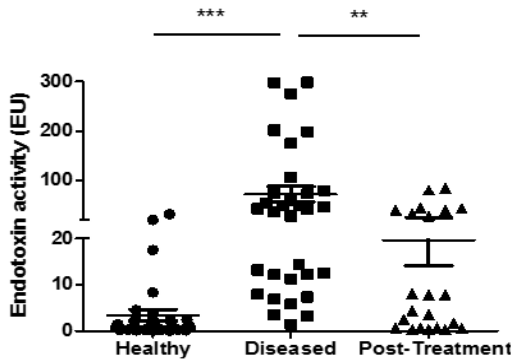

Fig. 2 Endotoxin activity of subgingival LPS extracts from individuals with healthy periodontium and chronic periodontitis patients before and after periodontal treatment. Lines represent mean values with SEM. (*** $p<0.001$; ** $p<0.01$).

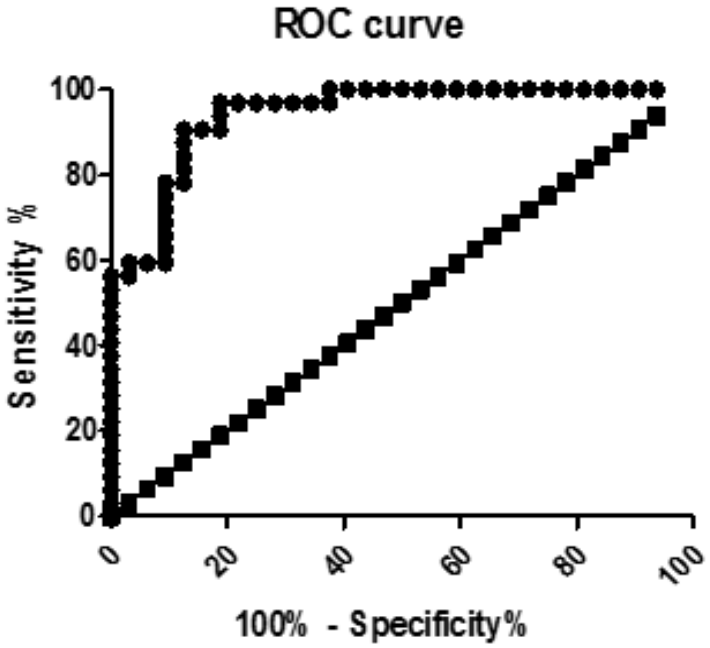

Fig. 3 The ROC curve for endotoxin activity (EU) in healthy and diseased patients as a biomarker for the disease status.

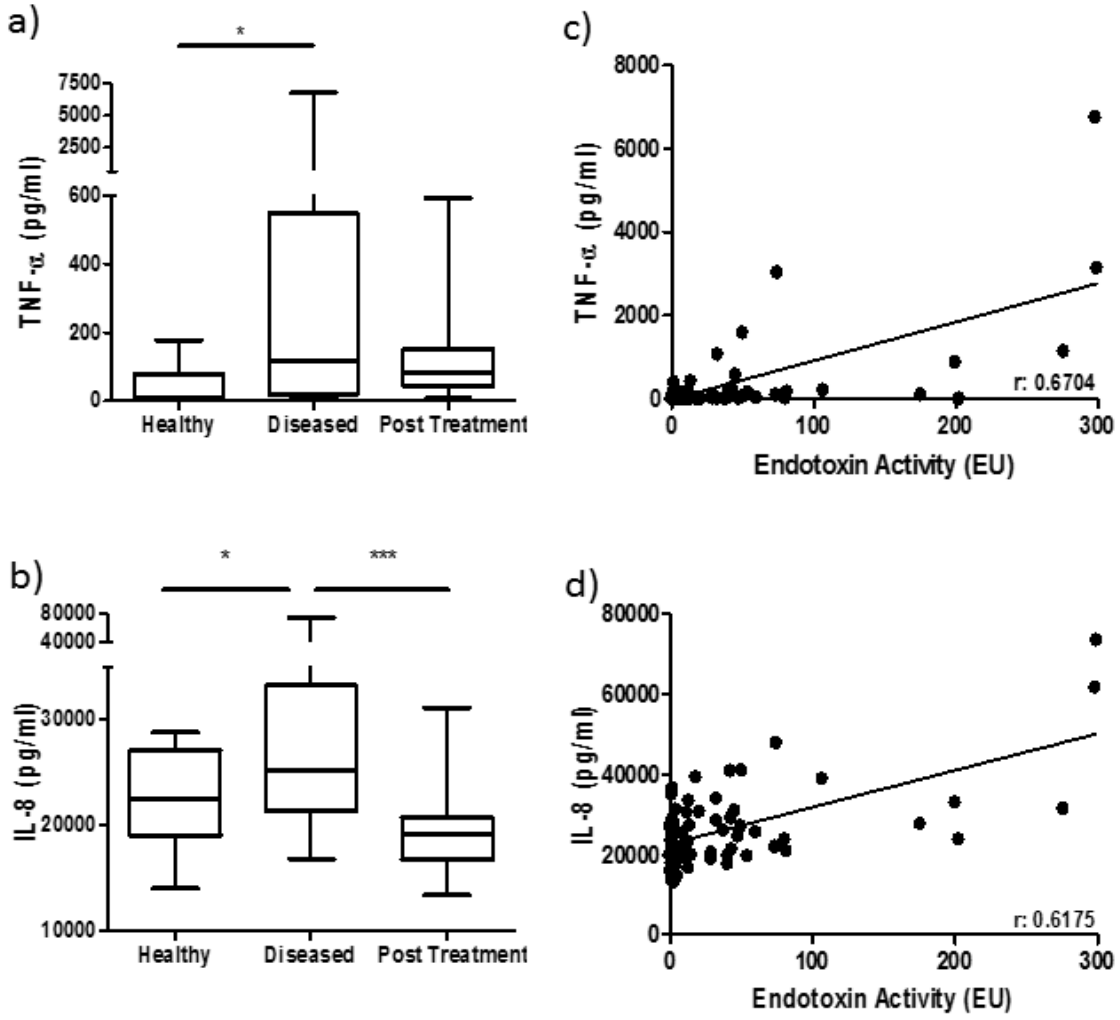

Fig. 4 The production of TNF-α (a) and IL-8 (b) by macrophages challenged with LPS extracts from healthy, diseased and post-treatment subgingival plaque samples for 18 hours. The correlation between TNF-α (c) and IL-8 levels (d) and subgingival endotoxin activity. (* $p<0.05$; *** $p<0.001$).